Resistance to Pesticin, Storage of Iron, and Invasion of HeLa Cells by Yersiniae†

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The independent abilities of *Yersinia pestis* to absorb exogenous pigments including hemin and Congo red (Pgm$^+$) and to produce the bacteriocin pesticin with genetically linked invasive enzymes (Pst$^+$) are established virulence factors of the species. Pst$^-$ Pgm$^+$ strains of *Y. pestis* are sensitive to pesticin (Pst^s), and mutation of these isolates to pesticin resistance (Pst^r) is known to result in concomitant conversion to Pgm$^-$. Wild-type cells of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are Pgm$^-$ but may be Pst^s; mutation of the latter to Pst^r also results in avirulence. In this study, typical Pgm$^-$ mutants of *Y. pestis* exhibited a dramatic nutritional requirement at 37°C but not 26°C for iron which could be fulfilled by either Fe^{3+} or hemin. Iron privation of Pgm$^-$ yersiniae resulted in formation of osmotically stable spheroplasts similar to those previously observed after exposure of Pst^s bacteria to pesticin. At 37°C, Pgm$^+$ organisms rapidly overgrew initially predominant Pgm$^-$ populations in iron-deficient medium. However, Pgm$^-$ isolates could undergo a second mutation that permitted successful competition with Pgm$^+$ cells in this environment. The mutation to Pst^r in *Y. pseudotuberculosis* and *Y. enterocolitica* did not promote a similar requirement for iron but rather prevented these organisms from penetrating HeLa cells. The ability to invade these nonprofessional phagocytes was not shared by Pgm$^+$ or Pgm$^-$ cells of *Y. pestis*.

Wild-type cells of *Yersinia pestis*, the causative agent of bubonic plague, absorbed exogenous hemin (26) or the dye Congo red (43) during growth at 26°C on solid media thereby forming intensely colored or pigmented colonies (Pgm$^+$). Mutational loss of this evident chromosomal function (17, 42) resulted in comparable growth as white colonies (26, 43), disappearance of a major outer membrane peptide (42), and marked reduction of virulence in mice by intraperitoneal (27) or subcutaneous (47) routes of injection. Typical cells of closely related *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* were Pgm$^-$ as judged by their growth as white colonies under conditions used to define pigmentation in *Y. pestis* (43; R. R. Brubaker, unpublished observations). Subsequent work showed that other pathogenic species including neisseriae, shigellae, *Vibrio*, and certain isolates of *Escherichia coli* were Pgm$^+$ as judged by their ability to absorb Congo red in environments distinct from that established as optimal for *Y. pestis* (5, 13, 33, 35); this determinant was correlated with the ability of shigellae to penetrate nonprofessional phagocytes (13).

Ability to produce the bacteriocin pesticin (2, 23, 24) and genetically linked (11, 31) coagulase and fibrinolysin (1) serves as an additional virulence factor (9) of *Y. pestis* (Pst$^+$). Loss of these functions, which are mediated by an approximate 6 megadalton plasmid (3, 17, 42), also promoted avirulence in mice by intraperitoneal and subcutaneous routes of injection (9). *Y. pseudotuberculosis* and *Y. enterocolitica* cells are Pst$^-$, but isolates of certain serotypes of these species (12, 36) and a few strains of *E. coli* (10, 18, 24) were also sensitive to the antibacterial action of pesticin (Pst^s). This lethal effect was catalyzed by the *N*-acetylglucosaminidase activity of the bacteriocin (16) which promoted conversion of Pst^s bacteria to osmotically stable spheroplasts (20). Expression of Pst^s in *Y. pestis* required loss of

immunity to the bacteriocin by mutation to Pst$^-$ (21) with retention of the Pgm$^+$ determinant (8). Subsequent mutation of these Pst$^-$ Pgm$^+$ organisms to pesticin resistance (Pst^r) resulted in selection for Pgm$^-$ isolates thereby permitting determination of a mutation rate from Pgm$^+$ to Pgm$^-$ of 10^{-5} (8). Analogous Pst^r mutants of *Y. pseudotuberculosis* and *Y. enterocolitica* were subsequently isolated and, like Pgm$^-$ and Pst$^-$ cells of *Y. pestis*, were of reduced virulence in mice via intraperitoneal and subcutaneous routes of injection (47).

Pgm$^+$ and Pst^s yersiniae were maintained in mouse liver, spleen, and lung, whereas Pgm$^-$ and Pst^r mutants were rapidly cleared from these organs, suggesting loss of a common mechanism of virulence (47). The Pgm$^+$ phenotype was assumed from the outset (7) to favor accumulation of iron in vivo (26, 27). However, attempts to demonstrate a selective advantage of Pgm$^+$ organisms in iron-deficient environments including serum (28) or 8-hydroxyquinoline-extracted medium (37) were not successful. Cells of *Y. pseudotuberculosis*, *Y. enterocolitica*, and both Pgm$^+$ and Pgm$^-$ *Y. pestis* utilized hemin as a sole source of iron and also accumulated Fe^{3+} by an inducible, siderophore-independent, cell-bound, high-affinity transport system (37). This finding suggested that all these organisms acquire iron by the same mechanism and that the Pgm$^+$ determinant merely serves as a mechanism to store the cation. The purpose of this report is to provide evidence indicating that avirulence associated with mutation to Pgm$^-$ in *Y. pestis* is indeed correlated with a significant lesion in iron accumulation. In contrast, avirulence caused by the analogous mutation to Pst^r in *Y. pseudotuberculosis* and *Y. enterocolitica* reflected loss of ability to invade nonprofessional phagocytes.

MATERIALS AND METHODS

Bacteria. All yersiniae used in this study were avirulent owing to loss of the ~45-megadalton plasmid that mediates the low-calcium response (3, 17). Unless stated otherwise,

TABLE 1. Properties of yersinia strains

Species	Strain	Pigmentation	Production of pesticin	Sensitivity to pesticin[a]	Plasmids (megadaltons)	Biotype[b] or serotype	Source or origin
Y. pestis	Kuma	+	+	I	6, 65	A	Manchuria
	KIM	+	+	I	6, 65	M	Iran
	G32	+	−	S	65	O	United States
	TS	+	+	I	6, 65	O	Java
	M23	+	+	I	6, 65	O	Nonencapsulated mutant of MP6
	A12	+	−	S	65	O	From strain A1122 (United States)
	Salazar	+	+	I	6, 65	O	Bolivia
	Yokohama	+	+	I	6, 65	A	Japan
	Kimberley	+	+	I	6, 65	O	Africa
	Dodson	+	−	S	65	O	Unknown
	K10	+	+	I	6, 65	M	Iran
	MP6	+	+	I	6, 65	O	Unknown
Y. pseudotuberculosis	PB1	−	−	S	None	I	England
	1	−	−	S	None	I	Unknown
	MD67	−	−	S	None	I	United States
	Hale	−	−	S	None	I	England
	Galligue	−	−	S	None	I	England
	Parkin	−	−	S	None	I	England
Y. enterocolitica	Wa	−	−	S	None	O:8	United States
	P76	−	−	S	None	O:8	United States
	E701	−	−	S	None	O:9	Canada
	E736	−	−	S	None	O:21	Canada

[a] S, Sensitive; I, immune.
[b] Biotypes are *antiqua* (A), *mediaevalis* (M), and *orientalis* (O).

organisms used in experiments were *Y. pestis* Kuma (37), *Y. pseudotuberculosis* PB1 (12), and *Y. enterocolitica* WA (36). Salient properties of these strains and other yersiniae are shown in Table 1. Pgm⁻ mutants of *Y. pestis* were obtained on Congo red agar (43) incubated at 26°C, and Pst^r mutants of *Y. pseudotuberculosis* and *Y. enterocolitica* were isolated on solid medium containing homogeneous pesticin (23) by the method previously described for determining the mutation rate of *Y. pestis* to Pgm⁻ (8). Streptomycin-resistant mutants were selected at 26°C for the ability to form colonies on tryptose-blood agar base (Difco Laboratories, Detroit, Mich.) containing the antibiotic (100 U/ml).

Cultivation. Slopes of tryptose-blood agar base were incubated for 1 day (*Y. pseudotuberculosis* or *Y. enterocolitica*) or 2 days (*Y. pestis*) at 26°C after inoculation from stock cultures of buffered glycerol maintained at −20°C as previously described (1). Resulting organisms were suspended and appropriately diluted in 0.033 M potassium phosphate buffer (pH 7.0) (phosphate buffer) for use as inocula of chemically defined medium (53), yielding an initial optical density at 620 nm of 0.1 (corresponding to about 10^8 viable bacteria per ml). A single transfer was made in this medium containing 50 μM Fe^{3+} or 20 to 50 μM hemin in Erlenmeyer flasks (10% [vol/vol]) aerated at 200 rpm on a model G76 gyratory water bath shaker (New Brunswick Scientific Co., Inc., Edison, N.J.). Organisms in late logarithmic growth were harvested by centrifugation (17,000 × *g* for 10 min at 4°C), washed once in phosphate buffer, and then suspended at an optical density of 0.1 in control medium containing iron as described above or in iron-deficient medium. These cultures were aerated as already defined and, in some experiments, were used to inoculate subsequent transfers when the optical density had increased to 1.0. Iron-deficient

medium was prepared by extraction with 8-hydroxyquinoline (50) and contained <0.3 μM iron as determined by flame absorption spectroscopy.

Host-cell invasion. HeLa cells were prepared in suspension and used in experiments with yersiniae essentially as previously described (15). The cells were routinely cultivated at 37°C under 5% CO_2 in Eagle minimal essential medium (Difco) with 5% fetal calf serum containing streptomycin (75 μg/ml) and potassium penicillin G (100 U/ml). After growth to a density of 5 × 10^5 cells per ml, antibiotics were removed by three washes in minimal essential medium with fetal calf serum, and 2.0 ml of the final suspension was placed in roller tubes. The latter then received 10^8 yersiniae washed once in phosphate buffer in 0.1 to 0.2 ml of the same buffer and were placed in a model TC-5 roller apparatus (New Brunswick) at 37°C. After infection for 2 h, extracellular organisms were killed by further incubation in the presence of kanamycin (75 μg/ml) for 4 h. Intracellular bacteria were then released by disruption of the HeLa cells with a Dounce homogenizer and determined by plating appropriate dilutions on tryptose-blood agar base. Results of preliminary studies showed that significant uptake, when it occurred, commenced after 1 h and ceased after 2 h of incubation (15).

RESULTS

The degree of adaptation to the culture medium and the extent of prior exposure to iron markedly influenced bacterial growth during subsequent iron privation. To control these variables, the organisms were cultivated for a single transfer with Fe^{3+} or hemin before washing and dilution for use as inocula. Pgm⁺ cells of *Y. pestis* were capable of evident unlimited growth thereafter at 37°C in iron-deficient
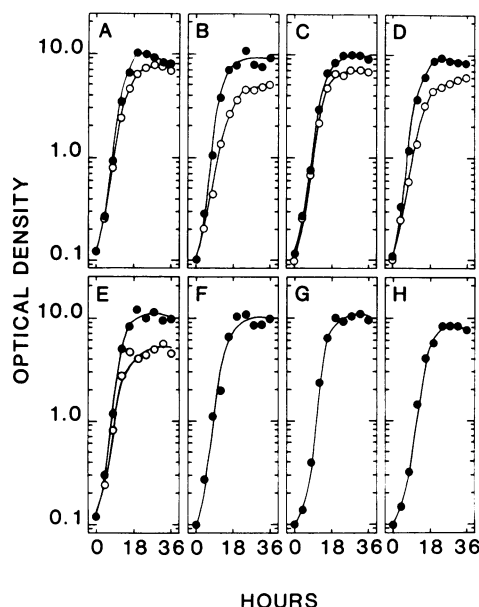
FIG. 1. Optical density of Pgm$^+$ cells of *Y. pestis* Kuma during the first (A), second (B), third (C), and fourth (D) transfer and of Pgm$^-$ mutants during the first (E), second (F), third (G), and fourth (H) transfer at 37°C in iron-deficient medium (O) or medium supplemented with 50 µM FeCl$_3$ (●). The organisms were initially grown with 50 µM Fe^{3+}, washed, and inoculated into the first transfers; subcultures were inoculated when the optical density of the parent culture was 1.0. Pgm$^-$ mutants failed to grow after the first transfer.

medium when prepared by this process after an initial transfer with Fe^{3+} (Fig. 1) or hemin (Fig. 2). Pgm$^+$ organisms also exhibited comparable generation times at 26°C in iron-deficient medium or in media supplemented with Fe^{3+} or hemin, although the latter promoted marked autoagglutination (data not shown). In contrast, Pgm$^-$ mutants exhibited only limited multiplication at 37°C upon transfer to iron-
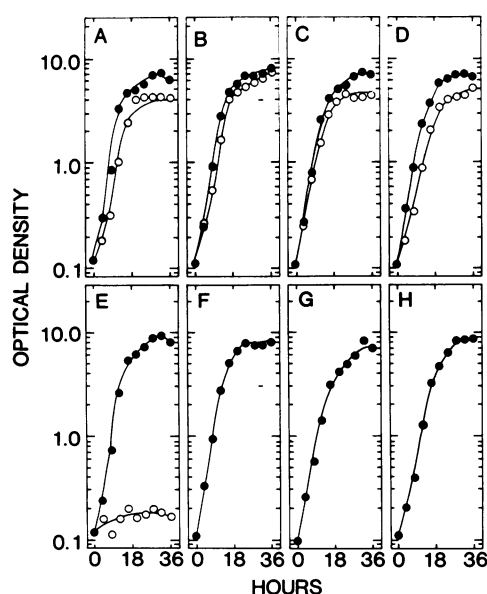

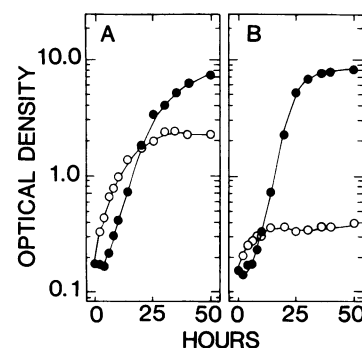FIG. 2. Same as Fig. 1 except that 50 µM hemin was used in place of Fe^{3+}.

FIG. 3. Optical density at 26°C (●) or 37°C (O) of Pgm$^-$ mutants of *Y. pestis* KIM in iron-deficient medium after a previous single transfer in the same medium at 26°C (A) or 37°C (B).

deficient medium after saturation of storage reservoirs with Fe^{3+} (Fig. 1) or hemin (Fig. 2). As shown below, growth of Pgm$^-$ organisms at 26°C in iron-deficient medium was equivalent to that observed at 37°C with added iron.

Pgm$^-$ mutants were cultivated for one passage with Fe^{3+} at 37°C and then subcultured once in iron-deficient medium at either 26 or 37°C. Regardless of the prior temperature of cultivation, a further transfer in iron-deficient medium at 26°C resulted in a lag followed by full-scale growth (Fig. 3). In contrast, similar incubation at 37°C resulted in restriction of growth, although significant residual multiplication occurred in the culture inoculated with organisms previously grown at 26°C. Accordingly, Pgm$^-$ mutants specifically exhibit an evident lesion in accumulation of iron at 37°C but not at 26°C. Although the nature of this temperature-dependent deficiency is unknown, it provides Pgm$^+$ bacteria in iron-deficient medium with a marked selective advantage. For example, an inoculum consisting of an artificial mixture of 99.95% Pgm$^-$ and 0.05% Pgm$^+$ organisms underwent a rapid and dramatic shift to an essentially pure Pgm$^+$ population during a single transfer in this environment (Fig. 4). These and related studies showed that the viability of Pgm$^-$ mutants at 37°C often decreased more rapidly in iron-deficient medium than did the optical density. This discrepancy was resolved upon observation of iron-deprived Pgm$^+$
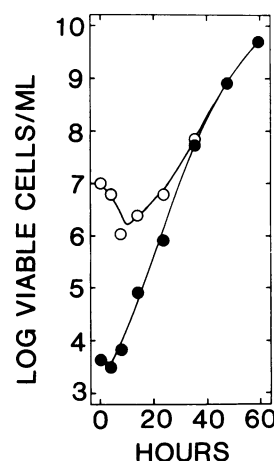

FIG. 4. Total cells (O) and Pgm$^+$ cells (●) of an artificial mixture initially composed of 99.95% streptomycin-sensitive Pgm$^-$ mutants and 0.05% streptomycin-resistant Pgm$^+$ organisms determined on tryptose-blood agar base and streptomycin agar, respectively.
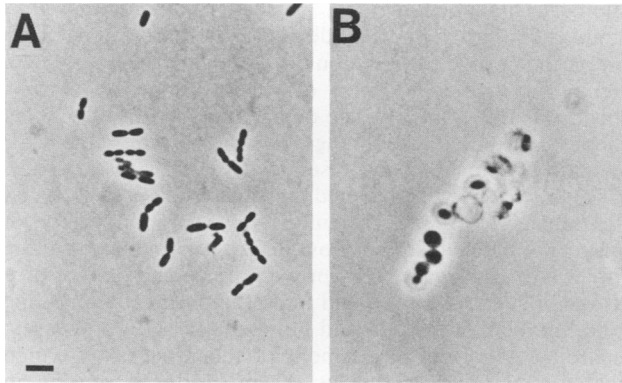
FIG. 5. Pgm⁺ cells of *Y. pestis* Kuma exhibiting normal morphology (A) and osmotically stable spheroplasts of Pgm⁻ mutants (B) after a single transfer in iron-deficient medium.

and Pgm⁻ organisms by light microscopy. The former exhibited normal morphology, whereas the latter underwent conversion to nonviable osmotically stable spheroplasts that nevertheless contributed to optical density (Fig. 5).

Other strains of *Y. pestis* were examined to determine whether the Pgm⁻-specific lesion in iron metabolism was a general property of the species. Of the 11 additional isolates tested, 8 resembled strain Kuma in that only Pgm⁺ organisms were capable of growth at 37°C without added iron (Table 2). Pgm⁻ mutants of the remaining three strains grew as well in this environment as did their Pgm⁺ parents. This finding suggested that Pgm⁻ bacteria typically exhibit the temperature-dependent defect since the exceptions pos-

TABLE 2. Doubling times and maximum optical density of Pgm⁺ and Pgm⁻ strains of *Y. pestis* at 37°C in chemically defined iron-deficient medium[a]

Strain	Pgm	Doubling time (min)	Maximum optical density
Kuma	+	110	8.160
	−	NG[b]	0.125
KIM	+	110	6.120
	−	NG	0.122
G32	+	150	7.960
	−	NG	0.137
TS	+	240	5.950
	−	NG	0.129
M23	+	170	6.340
	−	180	6.200
A12	+	220	7.000
	−	NG	0.119
Salazar	+	170	7.740
	−	NG	0.141
Yokohama	+	220	6.300
	−	NG	0.126
Kimberley	+	240	4.500
	−	240	5.550
Dodson	+	210	8.070
	−	230	7.080
K10	+	150	6.800
	−	NG	0.095
MP6	+	270	2.570
	−	NG	0.084

[a] Organisms were inoculated at an optical density of 0.1 after a single transfer in iron-deficient medium.
[b] NG, No growth (doubling time >24 h).

sessed various atypical characteristics and were of uncertain background (Table 1). However, the existence of these exceptions demonstrated that the ability to express the pigmentation reaction was not necessarily essential for growth at 37°C in iron-deficient medium. To further illustrate this point, Pgm⁻ cells of strain Kuma were cultivated at 37°C for 4 days without added iron at which time a subpopulation of otherwise typical Pgm⁻ organisms emerged that were capable of evident indefinite growth in this environment. These observations indicate that a suppressor mutation can promote multiplication of Pgm⁻ cells at 37°C in iron-deficient medium. Thus, acquisition of this ability does not reflect true reversion to Pgm⁺.

Since Pgm⁻ mutants are analogous to Pst^r mutants of *Y. pseudotuberculosis* and *Y. enterocolitica*, the latter were compared for ability to grow at 37°C in iron-deficient medium. Control Pgm⁻ cells of *Y. pestis* typically ceased division in this environment (Fig. 6A), whereas results obtained with *Y. pseudotuberculosis* were equivocal in that the Pst^r mutant grew more slowly than did the Pst^s parent but eventually achieved the same maximum optical density (Fig. 6B). No difference was detected between Pst^s and Pst^r cells of *Y. enterocolitica* (Fig. 6C). These findings indicated that mutation to Pst^r in *Y. enterocolitica* and probably *Y. pseudotuberculosis* did not result in significant loss of ability to accumulate iron as occurred with Pgm⁻ mutants of *Y. pestis*.

Accordingly, a search was initiated for other putative virulence functions present in Pst^s but not Pst^r mutants of *Y. pseudotuberculosis* and *Y. enterocolitica*. Attempts to demonstrate differences in adherence or ability to damage host cells were not successful, but the capability to penetrate nonprofessional phagocytes was significantly reduced in Pst^r mutants. This relationship is shown in Table 3 which shows that mutation of *Y. pseudotuberculosis* and *Y. enterocolitica* to Pst^r resulted in significant decreases in the ability to invade HeLa cells. Neither Pgm⁺ nor Pgm⁻ isolates of *Y. pestis* were capable of promoting detectable invasion of HeLa cells.

DISCUSSION

It is known that macrophages serve as target host cells for *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* (29, 44, 46). This relationship is emphasized by the observation that organisms of all three species are of highest virulence
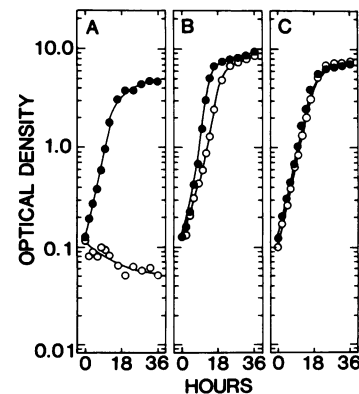

FIG. 6. Growth of Pgm⁺ (●) and Pgm⁻ (○) cells of *Y. pestis* Kuma (A), Pst^s (●) and Pst^r (○) cells of *Y. pseudotuberculosis* PBI (B), and Pst^s (●) and Pst^r (○) cells of *Y. enterocolitica* WA (C) during a second transfer in iron-deficient medium.

via intravenous injection (9, 47), in which immediate interaction occurs with fixed macrophages lining the blood vessels of the liver and spleen. Further evidence underscoring the importance to obtaining access to macrophages was the observation that virulence of Pgm⁻ and Pst⁻ mutants of *Y. pestis* (9, 47) and Pstʳ mutants of *Y. pseudotuberculosis* and *Y. enterocolitica* (47) could be phenotypically restored via intravenous injection. This finding suggests that these mutants are rapidly eliminated from the host by nonspecific mechanisms of defense after injection by peripheral routes but are able to cause acute disease after administration by a route that permits immediate uptake by macrophages (47). An important clue to the nature of these nonspecific mechanisms of host defense was the discovery that virulence of Pgm⁻, Pst⁻, and Pstʳ mutants could be fully restored via peripheral routes of infection by saturation of serum transferrin by injected iron (9, 27; unpublished obser-

vations). This phenomenon, originally made with Pgm⁻ mutants (27), first defined the debilitative effect of exogenous iron on the course of infectious diseases and has now been extended to include a variety of other pathogenic microorganisms (48, 52). Initially, the explanation of this effect was generally attributed to fulfilling a nutritional requirement for the cation which is tightly sequestered in vivo (51, 52). However, it is now established (48) that exogenous iron can also enhance virulence by interfering with a variety of nonspecific mechanisms of host defense including blocking synthesis of transferrin (34), preventing chemotaxis of professional phagocytes (49), and inhibiting intracellular killing by both oxidative (30, 40) and nonoxidative (19) processes. Accordingly, correct assessment of the process by which injected iron reverses the lesions in virulence present in Pgm⁻, Pst⁻, and Pstʳ isolates may be requisite to defining the nature of the virulence factors lost by these mutations.

Injected iron was assumed to enhance the virulence of Pst⁻ mutants of *Y. pestis* by inhibiting nonspecific mechanisms of host defense (7). Evidence favoring this possibility was the discovery that Pst⁻ mutants lacked coagulase and fibrinolysin and thus, after typical infection of peripheral tissues via fleabite, would be unable to initiate systemic infection (9, 11). Furthermore, it seems unlikely that the small 6-megadalton plasmid, known to encode pesticin, coagulase, fibrinolysin, and putative immunity plus maintenance functions, could also mediate iron transport activities. Accordingly, we suspect that the Pst⁺ determinant serves to enhance virulence by enabling the organisms to disseminate rapidly from peripheral foci of infection to favored niches within fixed macrophages of lymphatics and internal organs. This capability would enable the organisms to avoid lethal encounters with professional phagocytes other than macrophages. In contrast, the Pgm⁺ factor was predicted to increase virulence by promoting acquisition of iron in vivo (7, 26, 27). Nevertheless, the first direct evidence linking the Pgm⁺ determinant with transport of this cation is the observation reported here that typical Pgm⁻ cells do indeed exhibit an increased nutritional requirement for iron. This finding was not anticipated because a different medium, rendered iron deficient by the same process, did not reveal a difference in growth between Pgm⁺ and Pgm⁻ organisms (37). That medium, however, was prepared by avoiding incorporation of organic compounds capable of chelating significant Fe^{3+}. In contrast, the medium used in this study contained high levels of a number of biologically significant chelators, including 0.01 M citric acid (53). The presence of these ligands may account for the inability of Pgm⁻ mutants to multiply after removal of extractable Fe^{3+} since over 100 μM $FeCl_2$ was required for full-scale growth in the initial version (22) of the medium used.

An unexpected observation was that iron-deprived Pgm⁻ organisms underwent conversion to osmotically stable spheroplasts. Further study will be required to determine whether this phenomenon reflects the enzymatic activity of pesticin. If so, the dramatic loss of viability of Pgm⁻ mutants in iron-deficient medium may represent an inability to maintain immunity to the bacteriocin in the absence of iron. Alternatively, normally compartmentalized pesticin may be released after death in iron-deficient medium thereby promoting formation of spheroplasts. Evidence supporting this second possibility was the finding that the Pst⁺ determinant was not required for lethality of all Pgm⁻ mutants in iron-deficient medium. Indeed, the observation that three Pgm⁻ isolates and suppressor mutants of Pgm⁻ cells of strain Kuma grew normally in iron-deficient medium at 37°C also

TABLE 3. Infectivity for HeLa cells of Pgm⁺ and Pgm⁻ isolates of *Y. pestis*, Pstˢ and Pstʳ isolates of *Y. pseudotuberculosis*, and Pstˢ and Pstʳ isolates of *Y. enterocolitica*

Species	Strain	Phenotype	Infectivity[a]
Y. pestis	Kuma	Pgm⁺	<1
		Pgm⁻	<1
	KIM	Pgm⁺	<1
		Pgm⁻	<1
	G32	Pgm⁺	<1
		Pgm⁻	<1
	TS	Pgm⁺	<1
		Pgm⁻	<1
	M23	Pgm⁺	<1
		Pgm⁻	<1
	A12	Pgm⁺	<1
		Pgm⁻	<1
	Salazar	Pgm⁺	<1
		Pgm⁻	<1
	Yokohama	Pgm⁺	<1
		Pgm⁻	<1
	Kimberley	Pgm⁺	<1
		Pgm⁻	<1
	Dodson	Pgm⁺	<1
		Pgm⁻	<1
	K10	Pgm⁺	<1
		Pgm⁻	<1
	MP6	Pgm⁺	<1
		Pgm⁻	<1
Y. pseudotuberculosis	PB1	Pstˢ	14.5
		Pstʳ	1.0
	1	Pstˢ	18.0
		Pstʳ	1.3
	MD67	Pstˢ	7.5
		Pstʳ	<1
	Hale	Pstˢ	8.0
		Pstʳ	<1
	Galligue	Pstˢ	15.0
		Pstʳ	1.4
	Parkin	Pstˢ	14.0
		Pstʳ	1.0
Y. enterocolitica	WA	Pstˢ	8.4
		Pstʳ	2.7
	P76	Pstˢ	9.1
		Pstʳ	1.5
	E701	Pstˢ	7.1
		Pstʳ	<1
	E736	Pstˢ	8.3
		Pstʳ	1.0

[a] Number of viable bacteria per HeLa cell.

indicates that the Pgm⁺ determinant is not essential for transport of the cation in this environment. We are presently investigating the nature of this suppressor mutation and intend to determine whether it restores virulence of Pgm⁻ organisms via peripheral routes of injection.

Attempts to demonstrate a similar requirement for iron in Pstʳ mutants of *Y. pseudotuberculosis* and *Y. enterocolitica* were not successful. Accordingly, a comparative study was initiated to identify other deficiencies that might account for avirulence in these isolates. No significant differences were noted except in the ability to penetrate nonprofessional phagocytes as typified by HeLa cells. This capability was previously defined in *Y. pseudotuberculosis* (6) and *Y. enterocolitica* (13, 32, 45) and probably reflects that also reported for invasion of other types of nonprofessional phagocytes (25, 38). Further study will be required to determine whether this activity is identical to that transferred to *E. coli* K-12 as a single genetic locus from a pesticin-insensitive strain of *Y. pseudotuberculosis* (25). Similar penetration of host cells was not observed with *Y. pestis*, suggesting that this capability is only required by those yersiniae normally transmitted via the oral route of infection. Nevertheless, it is difficult to reconcile that selection for Pstʳ in *Y. pestis* results in recovery of Pgm⁻ mutants, whereas similar selection with Pstˢ isolates of *Y. pseudotuberculosis* or *Y. enterocolitica* permitted recovery of mutants unable to invade HeLa cells. Before this finding, we suspected that the pesticin receptor per se served as the hemin- and Congo red-binding component unique to Pgm⁺ cells of *Y. pestis*. This notion became untenable with the discovery that mutational loss of the pesticin receptor in the other yersiniae resulted in inability to invade HeLa cells. However, a strong possibility remains that the structural gene for the pesticin receptor is linked to genes encoding host-cell invasiveness in *Y. pseudotuberculosis* and *Y. enterocolitica* and linked to distinct genes in *Y. pestis* that mediate storage of iron. Development of methods permitting precise genetic analysis of chromosomal functions may be necessary to resolve this relationship.

The ability of *Y. pseudotuberculosis* and *Y. enterocolitica* to invade host cells would, like expression of the Pst⁺ factor, clearly provide protection against a variety of nonspecific mechanisims of host defense including destruction by professional phagocytes other than macrophages. However, this activity could also facilitate accumulation of iron as does the Pgm⁺ determinant. Evidence supporting this possibility is tenuous and dependent on the observations that other facultative intracellular parasites including neisseriae (41), listeriae (14), legionellae (39), and salmonellae (4) also utilize siderophore-independent mechanisms of iron transport in vivo possibly analogous to that first described for yersiniae (37). As initially suggested for legionellae (39), these findings are consistent with the hypothesis that available iron is extremely scarce in extracellular spaces but easily obtained in intracellular environments. If this assumption is correct, cells of *Y. pestis* may obtain and store needed iron via use of the Pgm⁺ factor, whereas the other yersiniae obtain the cation from intracellular reservoirs. In any event, we previously established that Pstʳ mutants of *Y. pseudotuberculosis* and *Y. enterocolitica* were of reduced virulence (47) and have now shown that this effect reflects the inability to invade nonprofessional phagocytes.

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